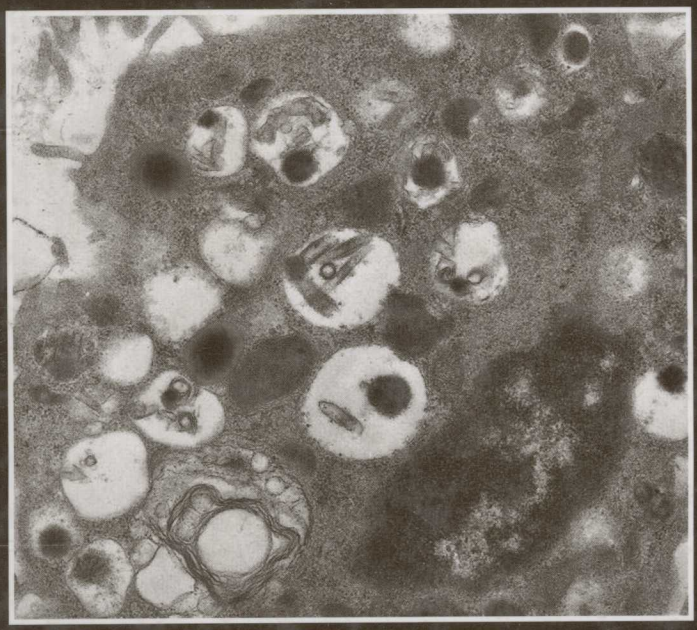
INTERNATIONAL REVIEW OF CELL AND MOLECULAR BIOLOGY

Edited by
Kwang W. Jeon



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Contents

CHAPTER ONE

Cell and Molecular Biology of Invadopodia

Giusi Caldieri, Inmaculada Ayala, Francesca Attanasio, *and* Roberto Buccione

Contents

Abstract

The controlled degradation of the extracellular matrix is crucial in physiological and pathological cell invasion alike. *In vitro*, degradation occurs at specific sites where invasive cells make contact with the extracellular matrix via specialized plasma membrane protrusions termed invadopodia. Considerable progress has been made in recent years toward understanding the basic molecular components and their ultrastructural features; generating substantial interest in invadopodia as a paradigm to study the complex interactions between the intracellular trafficking, signal transduction, and cytoskeleton regulation machineries. The next level will be to understand whether they may also represent valid biological targets to help advance the anticancer drug discovery process. Current knowledge will be reviewed here together with some of the most important open questions in invadopodia biology.

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1. Introduction

The ability of cells to invade the extracellular matrix (ECM) is essential in the response to injury, pathogen infection, embryogenesis, differentiation, neoangiogenesis, and also during tumor cell invasion and metastasis (Basbaum and Werb, 1996). In particular, migration-associated proteolytic degradation of the ECM is a common feature of cancer cells (Wolf and Friedl, 2009).

Invadopodia can be defined as stable actin-rich protrusions emanating from the ventral surface of invasive tumor or transformed cells, cultured on appropriate ECM substrates such as gelatin, fibronectin, collagen type I, collagen type IV, or laminin (Kelly *et al.*, 1994) and displaying focalized proteolytic activity toward the substrate (Chen, 1989; Mueller and Chen, 1991). Seminal work from the Chen and Mueller laboratories throughout the 1990s led to the identification of a number of molecular components including integrins, elements of signaling machineries, soluble and membrane-bound proteases (including matrix metalloproteases), and actin and actin-associated proteins such as cortactin and others (Bowden *et al.*, 1999; Chen, 1996; Monsky *et al.*, 1994; Mueller *et al.*, 1992; Nakahara *et al.*, 1997b). Considering the evidence accumulated to date, it appears that the biological function that can be specifically attributed to invadopodia is the degradation of ECM (Baldassarre *et al.*, 2003; Mizutani *et al.*, 2002; Nakahara *et al.*, 1997b; Yamaguchi *et al.*, 2005). Focal degradation of the ECM at invadopodia may thus very well recapitulate the initial steps of tumor cell invasion realized through the tight integration of the membrane remodeling, trafficking, and signaling machineries.

Invadopodia can be identified by the light microscope as roundish actin-rich structures at the ventral surface of cells (i.e., substrate face) that (1) are associated with sites of substrate degradation, (2) are not confined to the cell periphery, and (3) contain cortactin (or other actin related proteins, see below) and/or phosphotyrosine (Baldassarre *et al.*, 2006; Bowden *et al.*, 2006). Typical examples can be seen in Fig. 1.1. Other features that can be used to identify invadopodia, at least in some cell lines, include their location proximal to the Golgi complex (Baldassarre *et al.*, 2003), the central regulator for intracellular trafficking, and their extended half-life of up to 2 h or more (Baldassarre *et al.*, 2006; Yamaguchi *et al.*, 2005) as compared to related protrusive adhesions such as the podosomes (Linder, 2007; Linder and Aepfelbacher, 2003).

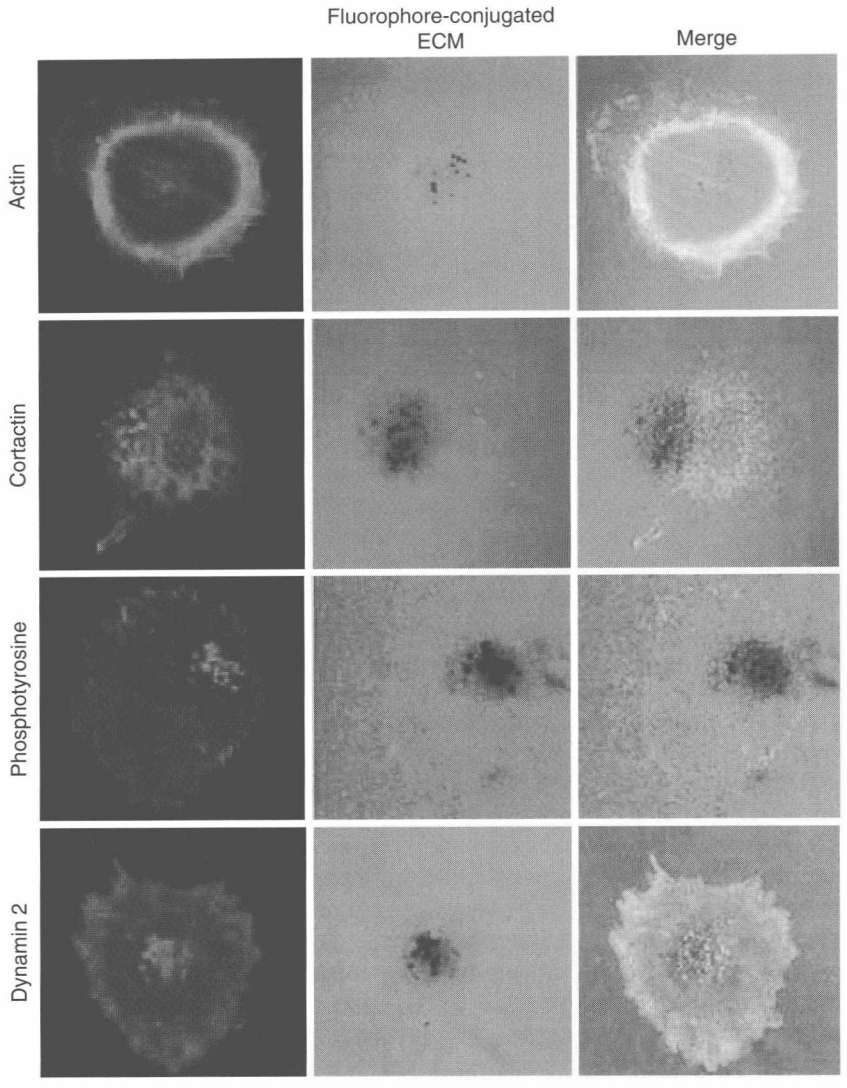

Figure 1.1 Identification of invadopodia. A375MM melanoma cells grown on FITC-conjugated gelatin (green) and then fixed and stained with Alexa 546-phalloidin and anti-cortactin, anti-phospho-tyrosine and anti-dynamin 2 antibodies (red). Invadopodia match with underlying areas of degradation. Merged images are also shown. (See Color Insert.)

2. Biogenesis, Molecular Components, and Activity

A number of reviews have comprehensively listed and discussed the components and pathways underlying the biogenesis and function of invadopodia (Ayala *et al.*, 2006; Gimona *et al.*, 2008; Linder, 2007;

Weaver, 2006). This section provides an updated bird's eye view of current knowledge, while highlighting those players who are currently the better characterized and addressing some of the most urgent questions in invadopodia biology. These include understanding the molecular and physicochemical cues that trigger invadopodia biogenesis, the signaling cascades transducing those cues to the membrane and cytoskeleton remodeling machineries, and how focal degradation of the ECM is established at invadopodia. Remarkably little is known on many of these aspects, although the field is witnessing a significant acceleration that no doubt will lead to significant advances in the near future in these and many other aspects of invadopodia biology.

2.1. Structure

The description of the ultrastructural features of invadopodia is still rather incomplete. In fact, an initial transmission electron microscopy observation on transformed fibroblasts (Chen, 1989) suggested that they are thin protrusions extending from the plasma membrane into the underlying ECM; this was later confirmed on a breast cancer-derived cell line (Bowden *et al.*, 1999). No further progress was reported for some years until a detailed ultrastructural analysis was performed on the melanoma cell line A375MM, based on a correlative confocal light electron microscopy technique whereby individual areas of ECM degradation with matching invadopodia were first identified at the light microscope and the very same analyzed at the electron microscope and reconstructed in three dimensions. In this study, invadopodia were shown to be originated from profound invaginations of the ventral surface of the plasma membrane. In general, such invaginations averaged 8 μm in width and 2 μm in depth. From within, many surface protrusions originated with diameters ranging from hundreds of nanometers to a few micrometers, and averaging 500 nm in length, which sometimes penetrated into the matrix (Baldassarre *et al.*, 2003). These protrusions were consistent with the originally described "invading" structures (Chen, 1989) but seemed to be part of more complex superstructures (Fig. 1.2). More recently, electron microscopy tomography experiments analyzed the connections between invadopodial protrusions and the cell body, showing them to be quite narrow (Fig. 1.3; Baldassarre *et al.*, 2006). Furthermore, additional evidence obtained by generating sections perpendicular to the substrate have confirmed the initial reconstructions (Beznoussenko, Caldieri, Giacchetti, and Buccione, unpublished date).

A consistent pattern arising from observations at the light and electron microscopy levels is the polarization and juxtaposition of the Golgi complex, the central secretory pathway processing unit of the cell, toward the invadopodial area (Caldieri, Giacchetti, Beznoussenko and Buccione, unpublished date; Baldassarre *et al.*, 2003), suggesting a tight relationship between proteolytic activity and membrane/protein transport (to be discussed below).

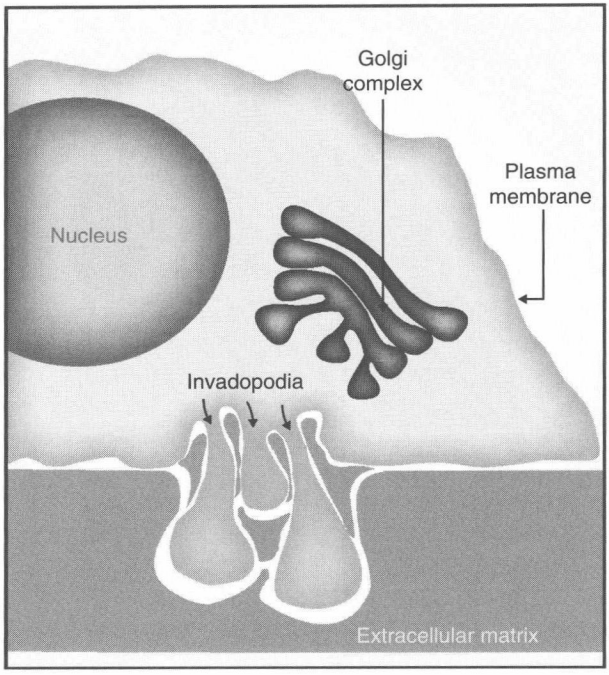

Figure 1.2 Schematic diagram of the invadopodial area. This is based on correlative light electron microscopy reconstructions on A375MM melanoma cells (Baldassarre *et al.*, 2003). Invadopodial protrusions originate from profound invaginations of the ventral surface of the plasma membrane; within the area delimited by the large invagination, large fragments of gelatin can often be seen. Also shown are the spatial relationships with the nucleus and the Golgi complex. Cartoon courtesy of Elena Fontana.

2.2. The cell–ECM interface

The engagement of cell surface integrins by substrate components is possibly the event that initiates invadopodia formation (Mueller *et al.*, 1999; Nakahara *et al.*, 1996, 1997b). The specific integrin combination that, when engaged, leads to invadopodia formation, might be cell-type dependent. In LOX melanoma cells, $\alpha_6\beta_1$ activation was found to promote Src-dependent tyrosine phosphorylation of p190RhoGAP, which in turn affected the actin cytoskeleton through the Rho family GTPases, thus activating membrane-protrusive and proteolytic activity, leading to invadopodia formation and cell invasion. The signaling pathways triggered by integrin engagement and leading to invadopodia formation and the molecular players involved in the cascade will be discussed later.

Integrins might also function as docking sites to spatially and temporally confine specific cellular activities and thus focalize the degradation process.

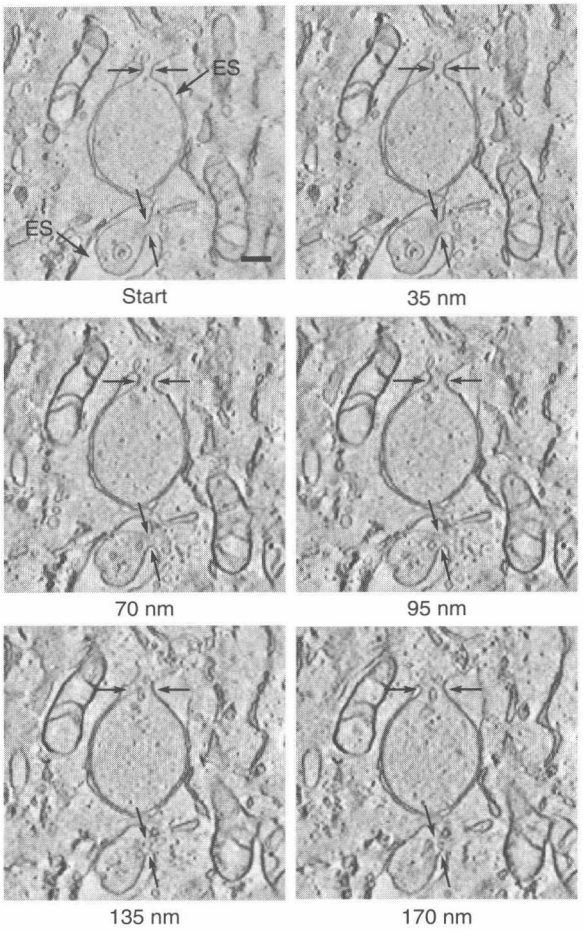

Figure 1.3 Invadopodia ultrastructure. Frame shots of an electron microscopy tomographic reconstruction of an invadopodial complex. Displacement along the vertical (z) axis is indicated in nanometers. The arrows indicate the narrow connections between the two invadopodial protrusions and the cell body. ES: extracellular space. Size bar is 200 nm. This image (Baldassarre *et al.*, 2006) is reproduced with permission from *The European Journal of Cell Biology.*

For example, collagen-induced $\alpha_3\beta_1$ association with the serine protease seprase was shown to drive the degradative activity of this gelatinolytic enzyme specifically at the tip of invadopodia (Artym *et al.*, 2002). An inhibitory anti-β_1 integrin antibody prevented the association between uPAR and seprase at invadopodia, suggesting a fundamental role for β_1 in the organization and targeting of proteases at sites of ECM degradation (Artym *et al.*, 2002). Integrins can also cooperate with the membrane-type 1

matrix metalloprotease (MT1-MMP) to localize and enhance proteolysis through the activation of matrix metalloprotease-2 (MMP2) (Deryugina *et al.*, 2001). Further, integrins might act at invadopodia by facilitating the clearing of partially degraded ECM by phagocytosis (Coopman *et al.*, 1998). In conclusion integrins are fundamental organizing centers to deploy the activity of various components at invadopodia. Research into the roles of integrins in invadopodia biology, given also potential implications for translational research, has been inexplicably stagnating for the last few years.

Recent research has shown that invadopodia formation can also be triggered/enhanced by soluble factors. For example, engagement of the EGF receptor with subsequent triggering of the signaling cascade leads to activation of the actin polymerization machinery (Yamaguchi *et al.*, 2005). At present a unifying model integrating ECM- and soluble ligand-derived activation is still lacking; furthermore, invadopodia formation does not necessarily require EGF receptor activation in many cell models.

2.3. Actin-remodeling machinery

2.3.1. Actin organization at invadopodia

The actin polymerization molecular machine based on the actin nucleator Arp2/3 and its activator N-WASP (Goley and Welch, 2006; Stradal and Scita, 2006) has been shown to be fundamental in the initiation and progression of the protrusive process leading to invadopodia formation. Hence, similar to lamellipodia, invadopodia rely on an Arp2/3-mediated branched actin meshwork, regulated and stabilized by cortactin. In general, however, the mechanisms generating the forces behind membrane remodeling and protrusion at invadopodia still need to be defined.

Two main hypotheses have recently been discussed (Vignjevic and Montagnac, 2008). In one, the constant growth of the branched actin meshwork would propel invadopodia into the underlying matrix, a mechanism similar to lamellipodia protrusions. Alternatively, following activation of the N-WASP/Arp2/3 system, and through the recruitment of actin bundling proteins, actin bundles could originate from the branched network, to win the stiffness of the substrate and to allow invadopodia protrusion, akin to filopodia formation. The evidence is still sparse and presents some inconsistencies possibly due to a combination of different experimental approaches and cell models. For instance, a FRET-based study showed that N-WASP is active at the base of the invadopodial protrusions in a rat mammary carcinoma cell line (Lorenz *et al.*, 2004). In another study, based on the A375MM human melanoma cell model, actin in actively degrading invadopodia was revealed as organized in very dynamic structures in which "head" (i.e., roundish, thicker) and "tail" (i.e., thinner, longer) sections were distinguishable (Baldassarre *et al.*, 2006), so that they explicitly resembled the actin-rich propelling structures associated with invading bacteria

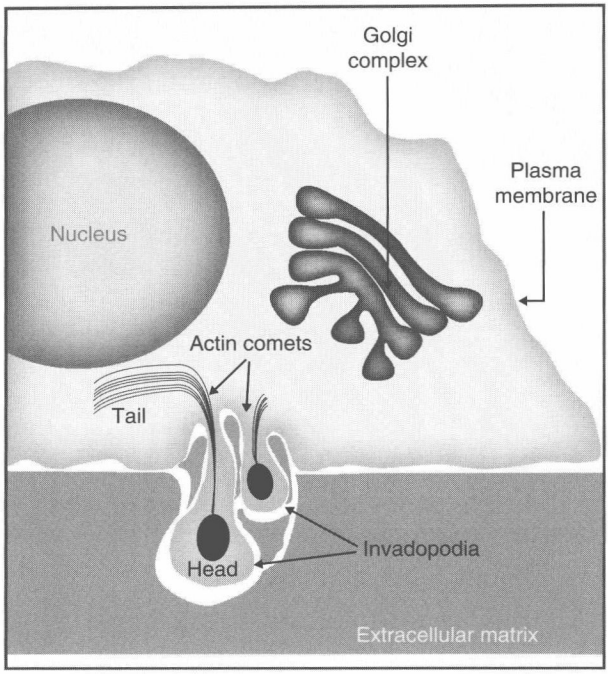

Figure 1.4 Actin comets at invadopodia. Graphical representation of actin tail structures contained within invadopodial protrusions. This is inferred from the previously published morphological descriptions of invadopodia (Baldassarre *et al.*, 2003, 2006). Where the nucleus "constrains" the structures, invadopodia-associated actin tails bend over and allow detection of a quasi-rotatory motion. Cartoon courtesy of Elena Fontana.

(i.e., actin comets or tails) (Cudmore *et al.*, 1995; Gouin *et al.*, 2005) with the striking difference that at invadopodia, the head sections remained stationary while the tails moved around continuously in a quasi-circular motion (Fig. 1.4). In this study, Arp2/3 and N-WASP were localized to the whole actin tail structure and to the "head" section, respectively, suggesting actin branching at the tip of the invadopodial protrusion (Baldassarre *et al.*, 2006). The main components of the actin polymerization machinery acting at invadopodia will be discussed below.

2.3.2. The Arp2/3 complex

Arp2/3 is a seven-protein complex that contains two related proteins (Arp2 and Arp3) and five unique polypeptides (ARPC1-5) (Goley and Welch, 2006). The complex presents little biochemical activity on its own, but when activated, initiates the nucleation of a new actin filament that emerges from an existing one in a y-branch configuration with a regular 70° angle

(Mullins *et al.*, 1998). As such, Arp2/3 is responsible for the actin rearrangements that govern the formation of lamellipodia and filopodia and, in general, cell motility (Goley *et al.*, 2004).

Arp2/3 has been repeatedly localized at invadopodia (Baldassarre *et al.*, 2006; Yamaguchi *et al.*, 2005). Also, the downregulation of a single subunit by siRNA and the overexpression of N-WASP mutants (see below) that lack the sequence required for Arp2/3-binding or the CA domain of N-WASP that competes for the binding to Arp2/3, resulted in inhibition of invadopodia formation (Yamaguchi *et al.*, 2005). Altogether the results provide evidence of the requirement of Arp2/3 for invadopodia biogenesis and ECM degradation. A dysfunction in the Arp2/3 complex might also be associated with cancer metastasis, which relies on the ability of cells to migrate away from the primary tumor. Indeed, the expression of Arp2/3 together with N-WASP and other factors related with cell motility is upregulated in some tumor tissues and invasive cells (Otsubo *et al.*, 2004; Semba *et al.*, 2006).

2.3.3. N-WASP

The WASP family of proteins includes hematopoietic WASP, ubiquitous N-WASP, and WASP family verprolin homologous (WAVE) proteins (WAVE1, WAVE2, and WAVE 3) (Bompard and Caron, 2004). WASP was originally identified as the causative gene product for the hereditary X-linked Wiskott–Aldrich syndrome, characterized by thrombocytopenia and immunodeficiency. WASP and N-WASP are direct effectors of Cdc42, whereas WAVE proteins play a major role in Rac-induced actin dynamics. All these proteins are implicated in a variety of cellular processes such as formation of membrane protrusions, vesicular trafficking and motility of pathogens. The family possesses a common C-terminal catalytic verprolin homology, cofilin homology or central, acidic (VCA) domain for the activation of the Arp2/3 complex, which induces rapid actin polymerization and generates a branched network of actin filaments (Mullins *et al.*, 1998; Welch *et al.*, 1998).

The expression of dominant negative mutants of N-WASP, unable to activate the Arp2/3 complex, suppressed invadopodia formation in v-Src-transformed 3Y1 rat fibroblasts (Mizutani *et al.*, 2002). Also, activation of N-WASP was detected at actively degrading invadopodia (Lorenz *et al.*, 2004). Furthermore, N-WASP depletion in metastatic rat adenocarcinoma MTLn3 cells reduced their ability to form invadopodia whereas WAVE1 and WAVE2 knockdown cells formed invadopodia as efficiently as control cells (Yamaguchi *et al.*, 2005). These results suggested that N-WASP in implicated in invadopodia biogenesis and as a consequence, ECM degradation.

Many upstream activators of N-WASP such as Grb2, Nck, WASP-interacting SH3 protein (WISH) and WASP-interacting protein (WIP) (Carlier *et al.*, 2000; Fukuoka *et al.*, 2001; Rohatgi *et al.*, 2001) have been identified. The contribution of many of these molecular components in

invadopodia biogenesis has also been investigated. Invadopodia formation and degradation activity was markedly suppressed in the same cells by depletion of Nck but not Grb2 (Yamaguchi *et al.*, 2005). WIP is thought to link Nck and N-WASP to induce actin polymerization (Benesch *et al.*, 2002). The ectopic expression of the wild-type form showed that this protein accumulated significantly at invadopodia. Additionally, transfection of the N-WASP binding domain (WBD) of WIP showed a marked reduction of invadopodia formation (Yamaguchi *et al.*, 2005). These data suggested that the interaction between N-WASP and WIP was needed for invadopodia formation. An additional note of interest is that similar to Arp2/3, N-WASP is overexpressed in several cancer types (Yamaguchi and Condeelis, 2007).

2.3.4. Cortactin

Cortactin is an actin-binding protein involved in the coordination of cell migration, cytoskeleton remodeling, and intracellular protein transport (Ammer and Weed, 2008; Olazabal and Machesky, 2001). Human cortactin is encoded by the CTTN gene on chromosome 11q13, frequently amplified in breast, head and neck squamous carcinoma and bladder cancer (Bringuier *et al.*, 1996; Schuuring, 1995). Cortactin features an N-terminal acidic domain containing a conserved DDW motif that binds and weakly activates the Arp2/3 complex (Uruno *et al.*, 2001). This is followed by a variable number of 37 amino acid repeats (depending on the splice variant; van Rossum *et al.*, 2003), constituting the actin-binding domain of which only the fourth is required for F-actin-binding activity, and has been suggested to stabilize the newly created branches between filaments (Weaver *et al.*, 2001, 2003). Cortactin was originally identified as a major substrate of Src (Wu *et al.*, 1991) and later found to be tyrosine-phosphorylated in response to stimuli that induce remodeling of the actin cytoskeleton, as for instance FGF, EGF, or integrins (Vuori and Ruoslahti, 1995; Zhan *et al.*, 1993). In particular, phosphorylation of Y421, 466, and 482 in the proline-rich domain (PRD) has been shown to be required for motility of endothelial cells (Huang *et al.*, 1998) and metastatic dissemination of breast carcinoma cells (Li *et al.*, 2001).

Cortactin has also been shown to play a fundamental role in invadopodia formation and function. After an earlier study showing that microinjection of antibodies against cortactin blocked matrix degradation at invadopodia (Bowden *et al.*, 1999), other reports have corroborated and extended this finding with diverse approaches including RNA interference and functional domain analysis. These results have highlighted the importance of cortactin phosphorylation by Src in invadopodia function (Artym *et al.*, 2006) and suggested that cortactin might function by regulating metalloprotease secretion at sites of ECM degradation (Clark *et al.*, 2007). One study investigated the role of various protein kinases in cortactin function at invadopodia,